An Investigation of the Molecular Properties and Stability of Intermediates of Proenkephalin in Isolated Bovine Adrenal Medullary Chromaffin Granules*

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An antiserum to a synthetic peptide corresponding to residues 95–117 of bovine proenkephalin recognizes all the major intermediates of this prohormone in bovine adrenal medulla (Birch, N. P. and Christie, D. L. (1986) J. Biol. Chem. 261, 12213–12221). This antiserum enabled an investigation of the stability and molecular properties of intermediates in the processing of proenkephalin in bovine adrenal medullary chromaffin granules. Intact and hypotonic lysates of chromaffin granules were incubated at 37 °C and the stability of intermediates assessed by gel filtration followed by radioimmunoassay and gel electrophoresis in combination with immunoblotting. Processing was slow in intact granules compared with incubations of hypotonic lysates which resulted in the selective cleavage of an M, 27,000 intermediate and increases in the amounts of immunoreactivity of lower molecular weight. Protease inhibitors increased the stability of the 27-kilodalton intermediate, the most effective being p-chloromercuribenzoate. Preliminary evidence was obtained for the regulation of the processing of this intermediate by soluble factors present in chromaffin granules. It appears that membrane-associated intermediates of proenkephalin are relatively stable, although analysis of soluble immunoreactivity released during the incubation of chromaffin granule membranes showed a decrease in the 27-kilodalton intermediate and increased amounts of lower molecular weight intermediates. Analysis of hypotonic lysates by two-dimensional gel-electrophoresis showed that proenkephalin intermediates exhibit significant microheterogeneity. It will be important to compare the products of proenkephalin generated by purified proteases with a putative role in the processing of this prohormone with the properties of endogenous intermediates as revealed in this study.

Most biologically active peptides are synthesized as part of larger precursor molecules (1), and the conversion of these prohormones to active peptides requires packaging into secretory granules (2) and limited proteolysis by highly specific proteases (3). Bovine adrenal medullary chromaffin granules contain relatively large amounts of EC polypeptides which are intermediates in the biosynthesis of enkephalins (4). The structure of the enkephalin precursor molecule proenkephalin has been established by sequencing the DNA complementary to the mRNA coding for this prohormone (5, 6). Bovine proenkephalin contains four copies of Met-enkephalin and one copy each of Leu-enkephalin, Met-enkephalin-Arg⁶-Gly⁷-Leu⁸, and Met-enkephalin-Arg⁶-Phe⁷. The sequence of each enkephalin peptide is bordered by paired basic amino acid residues (-Lys-Lys-, -Arg-Lys-, and -Arg-Arg-) which are believed to act as recognition sequences for processing enzymes. Proteases that cleave EC peptides at double basic amino acid residues (7, 8) and carboxypeptidase B-like activities (9, 10) have been identified in bovine adrenal chromaffin granules, and progress has been made in the isolation of these putative processing enzymes (11, 12). However, the processing of proenkephalin is complicated by evidence indicating that the processing of EC peptides in intact bovine chromaffin granules is slow (13), accounting for the dominance of high molecular weight EC peptides in this tissue (4).

Most studies of processing have been concerned with the generation of enkephalin peptides from small EC peptides. There is a need for studies concerned with the processing of high molecular weight intermediates of proenkephalin, but this has been hindered by the availability of suitable substrates and difficulties regarding the mode of assay. We have shown that antisera generated to a synthetic peptide corresponding to residues 95–117 of bovine proenkephalin recognize all the major intermediates of proenkephalin in bovine adrenal medulla (14). In the present work this antiserum has been used to characterize and to assess the stability of intermediates of proenkephalin in intact chromaffin granules as well as soluble and membrane components of the granules after hypotonic lysis.

**EXPERIMENTAL PROCEDURES**

**Preparation of Anti-proenkephalin Sera**

Antisera were raised against a synthetic peptide corresponding to residues 95–117 of bovine proenkephalin, proenkephalin-(95–117), as described previously (4). Antiserum RB-4 was used throughout the present study.

**Preparation of Bovine Adrenal Medullary Chromaffin Granules**

Chromaffin granules were prepared as described in Ref. 14. Pellets of chromaffin granules (12 pellets were obtained from 50 g of adrenal medullary tissue after the final ultracentrifugation step) were lysed by the addition of 5 mm Tris succinate, pH 5.9, followed by freezing and thawing (4 °C). The suspension was centrifuged at 100,000 × g

*The abbreviations used are: EC, enkephalin-containing; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride.

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for 45 min (Beckman 50 Ti rotor, 40,000 rpm), and the supernatant (chromaffin granule lysate) was used for experiments concerned with the processing of proenkephalin. Some experiments detailed below utilized either intact granules or chromaffin granule membrane pellets obtained after centrifugation of lysed granules.

Characterization of the Stability of Proenkephalin in Intact Granules and Chromaffin Granule Lysates by Gel Filtration

Intact chromaffin granules were resuspended in 1.5 ml of 0.1 M sodium phosphate, 0.3 M sucrose, pH 6.5 (13), and incubated at 37 °C. Aliquots (250 µl) were removed at various times and added to 1.75 ml of 1 M acetic acid, 2 mM PMSF (preheated to 95 °C). Samples were heated at 95 °C for 30 min, centrifuged (20,000 g, 30 min, 4 °C), and subjected to gel filtration.

Hypotonic lysates of chromaffin granules (5 ml, in 5 mM Tris succinate, pH 5.9) were incubated at 37 °C. Aliquots were removed at various times and subjected to heating in 1 M acetic acid, 2 mM PMSF and gel filtration as described for intact granules. All samples were chromatographed on a Sephadex G-75 column (1.4 X 80 cm) run in 1 M acetic acid at 4 °C. Aliquots of fractions were diluted in radioimmunoassay buffer and the proenkephalin immunoreactivity determined using the proenkephalin-(9-17) radioimmunoassay (14). The results were expressed in terms of nanograms of proenkephalin immunoreactivity/mg of protein present in samples of intact chromaffin granules or lysate. Protein was estimated by the method of Markwell et al. (15).

Characterization of the Stability of Intermediates of Proenkephalin in Intact Chromaffin Granules, Chromaffin Granule Lysate, and Chromaffin Granule Membranes by SDS-PAGE Combined with Immunoblotting

SDS-PAGE and Immunoblotting—Intermediates in the processing of proenkephalin were characterized by gel electrophoresis and immunoblotting using the same conditions as those described previously (14). To investigate the relative staining associated with the different molecular weight intermediates, serial dilutions of lysate samples were subject to electrophoresis and immunoblotting. The immunoblots were scanned by densitometry (625 nm, Helena) after treatment with toluene to render the nitrocellulose transparent.

Incubation of Chromaffin Granule Lysate—Samples of lysate were incubated at 37 °C, and aliquots (5 µl) were removed at various times (usually 1-3 and 15 h after incubation) and freeze-dried. In some experiments, incubation of chromaffin granule lysates was carried out in the presence of 2 mM PMSF, 1 mM PCMB, 1 mg/ml soybean trypsin inhibitor, and 1 mM leupeptin. Prior to electrophoresis, samples were boiled for 5 min in gel electrophoresis buffer (0.0625 M Tris-HCl, pH 6.8, 2.3% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% glycerol, 0.001% (w/v) bromphenol blue).

Some experiments were carried out with chromaffin granules which had been lysed in one-tenth the normal volume of hypotonic buffer, termed "small-volume" lysates. These samples were incubated at 37 °C and aliquots (5 µl) taken for analysis by gel electrophoresis and immunoblotting. Parallel experiments were carried out in which the "small-volume" lysate was diluted 10 times prior to incubation.

Incubation of Intact Chromaffin Granules and Chromaffin Granule Membranes—Intact chromaffin granules were incubated at 37 °C in 0.1 M sodium phosphate, 0.3 M sucrose, pH 6.5, at 37 °C (13). Aliquots (25 µl) were taken at various times, diluted with 1 volume of two-times concentrated gel electrophoresis buffer, and analyzed as described above. In some experiments, intact granules after incubation were lysed by dilution with 5 mM Tris succinate, pH 5.9, followed by freezing and thawing, and the soluble and membrane components obtained after centrifugation were each analyzed by gel electrophoresis and immunoblotting.

Incubations of chromaffin granule membrane fractions were carried out in 5 mM Tris succinate, pH 5.9, at 37 °C. At various times soluble and membrane components, recovered by ultracentrifugation, were each analyzed by gel electrophoresis and immunoblotting.

Characterization of Proenkephalin Intermediates in Chromaffin Granule Lysates by Two-dimensional Gel Electrophoresis

Samples of chromaffin granule lysate (100 µl) were freeze-dried and dissolved in 100 µl of lysis buffer (16). Two-dimensional electrophoresis was essentially as described in Ref. 16 with the modification that the concentration of Ampholines (LKB) in the isoelectric focusing gel was increased to 2.8% consisting of 2%, pH range 3.5 to 5 and 0.8%, pH range 3.5 to 10 (17). The second dimension SDS-polyacrylamide gel was the same as that used for the analysis of proenkephalin intermediates. Protein bands were visualized by staining with Coomassie Blue and proenkephalin intermediates were detected by immunoblotting.

RESULTS

In preliminary studies the stability of proenkephalin intermediates following incubation of both intact and hypotonic lysates of bovine chromaffin granules at 37 °C was assessed by gel filtration (Fig. 1A and B). Incubation of intact granules for 15 h resulted in little change in the higher molecular weight components (pools 1 and 2) although a considerable increase of low molecular weight (pool 3) material was observed. By comparison, incubation of hypotonic lysates resulted in increases in the relative amounts of immunoreactivity associated with pools 2 and 3 (Fig. 1B). The results obtained for samples which had been incubated for 4 h were similar to those obtained at 15 h (data not shown). The molecular distribution of proenkephalin immunoreactivity of both intact granules and hypotonic lysates was similar in samples prior to incubation.

![Fig. 1. Analysis of the stability of proenkephalin intermediates in intact chromaffin granules and chromaffin granule lysate by gel filtration. A, intact chromaffin granules, suspended in 0.1 M sodium phosphate, 0.3 M sucrose, 0.1% 2-mercaptoethanol, and B, chromaffin granule lysate in 5 mM Tris succinate, pH 5.9, were incubated at 37 °C and samples taken at various times and extracted with heated 1 M acetic acid, 2 mM PMSF and subjected to gel filtration on a column (1.4 X 80 cm) of Sephadex G-75 run in 1 M acetic acid. Aliquots of fractions were diluted with assay buffer and the proenkephalin immunoreactivity determined by radioimmunoassay. The elution profile of proenkephalin immunoreactivity obtained before (dashed) and after (+) incubation of these fractions is shown. Protein bands were visualized by staining with Coomassie Blue and proenkephalin intermediates were detected by immunoblotting.](image-url)
Further characterization of the processing of proenkephalin in hypotonic lysates of chromaffin granules utilized the higher resolving power of SDS-PAGE in combination with immunoblotting. Analysis of hypotonic lysates at 37 °C showed a 27-kDa intermediate of proenkephalin to disappear over a 2-h period at 37 °C. In contrast the 20.5- and 16.5-kDa immunoreactive species appeared to be relatively stable (Fig. 2, a and b). Densitometric scans of immunoblots showed a linear relationship between the amount of lysate applied to the gel and the staining associated with the 27-kDa immunoreactive species but not for the 20.5- and 16.5-kDa components (data not shown). The higher levels of these components in lysate samples relative to the 27-kDa intermediate meant that it was also necessary to analyze lysate samples after dilution. Analysis of diluted samples of lysate from the experiment of Fig. 2a confirmed that the 20.5- and 16.5-kDa components were relatively stable intermediates (Fig. 2b). These results were supported by densitometric scans of the immunoblots of Fig. 2 (data not shown). In contrast to the results with hypotonic lysate, analysis of intact chromaffin granules after incubation at 37 °C indicated that little apparent processing of the 27-kDa intermediate of proenkephalin had occurred (Fig. 3). This result was confirmed by densitometry (data not shown). It appeared that processing may be regulated in intact granules. To see if this was concentration-dependent, an experiment was carried out in which chromaffin granules were lysed in one-tenth the usual volume of hypotonic buffer prior to incubation at 37 °C. No processing of the 27-kDa intermediate was observed; however, incubation of a sample of the small-volume lysate diluted to give a concentration the same as that of normal lysate resulted in the disappearance of the 27-kDa intermediate of proenkephalin (Fig. 4).

A range of protease inhibitors was tested for their effect on the stability of the 27-kDa intermediate during incubation of chromaffin granule lysates by electrophoresis, immunoblotting, and densitometry (data not shown). Treatment with p-chloromercuribenzoate, PMSF, and soybean trypsin inhibitor resulted in 84, 49, and 62% of the 27-kDa intermediate of proenkephalin remaining after 2 h of incubation at 37 °C. By comparison, incubations without these inhibitors or containing leupeptin resulted in the complete disappearance of the 27-kDa intermediate.

It was also of interest to examine the stability of proenkephalin associated with chromaffin granule membranes. Both membrane and soluble components were relatively stable during the incubation of intact granules (Fig. 5A). Membrane-associated forms of proenkephalin were also stable during incubation of washed chromaffin granule membranes (Fig. 5B). However, analysis of material released into the incubation buffer from the membranes indicated that the 27-kDa component appeared to decrease with time and that this was accompanied by the appearance of new bands of 23.3- and 20.5-kDa and a significant increase in the levels of 16.5-kDa immunoreactive material (Fig. 5B).

We have also examined the molecular forms of proenkephalin present in hypotonic lysates of bovine adrenal medullary chromaffin granules by two-dimensional gel electrophoresis and immunoblotting. The results of incubations of "small-volume" lysate (a) and "small-volume" lysate diluted 10-fold prior to incubation (b) are shown. The molecular masses of the major immunoreactive forms are shown.

**Fig. 2. Analysis of the stability of proenkephalin intermediates in chromaffin granule lysate by SDS-PAGE and immunoblotting.** Chromaffin granule lysate was incubated at 37 °C in 5 mM Tris succinate, pH 5.9, and samples (25 μl) were removed at the times indicated and analyzed by electrophoresis and immunoblotting, a, analysis of lysate samples without dilution and b, analysis of lysate samples after 16-fold dilution.

**Fig. 3. Analysis of the stability of proenkephalin intermediates in intact chromaffin granules by SDS-PAGE and immunoblotting.** Intact chromaffin granules, suspended in 0.1 M phosphate buffer, 0.3 M sucrose, 0.1% 2-mercaptoethanol, pH 6.5, were incubated at 37 °C, and samples (25 μl) were removed at the times indicated for electrophoresis and immunoblotting. The molecular masses of the major intermediates from proenkephalin are shown.

**Fig. 4. Effect of the concentration of chromaffin granule lysate on the stability of the 27-kDa intermediate of proenkephalin.** Chromaffin granules were lysed in one-tenth the usual volume of hypotonic buffer (5 mM Tris succinate, pH 5.9) and divided into 2 aliquots. One of these was diluted 10-fold with buffer and both were incubated at 37 °C. Samples were removed at various times and characterized by SDS-PAGE and immunoblotting. The results of incubations of "small-volume" lysate (a) and "small-volume" lysate diluted 10-fold prior to incubation (b) are shown. The molecular masses of the major immunoreactive forms are shown.
In previous work we showed that an antiserum to a synthetic peptide corresponding to residues 95–117 of bovine proenkephalin recognizes all the major intermediates of proenkephalin in bovine adrenal medulla (14). Analysis of the intermediates of proenkephalin present in intact chromaffin granules by both gel filtration and immunoblotting indicated that intermediates were relatively stable although some formation of low molecular weight proenkephalin immunoreactivity occurred. This is consistent with a previous report which showed that the processing of high molecular weight EC proteins was slow in intact granules, with an estimated half-life of 6–8 days (13). We found that more extensive processing occurred in hypotonic lysates of chromaffin granules, and the results of gel filtration were consistent with cleavage of the high molecular weight pool 1 material (contains 27- and 20.5-kDa material) which would account for the increase in proenkephalin immunoreactivity associated with pool 2. The total immunoreactivity ng/mg protein also increased on incubation, an observation which can be explained by the fact that pool 2 immunoreactivity is more potent in the radioimmunoassay than pool 1 material (14). Analysis of SDS-PAGE in combination with immunoblotting showed the selective and rapid cleavage of a 27-kDa proenkephalin intermediate. By comparison, the 20.5- and 16.5-kDa intermediates appeared to be relatively stable.

Immunoblotting with anti-proenkephalin-(95–117) serum enabled the processing of the 27-kDa intermediate of proenkephalin to be studied in a semiquantitative manner. Cleavage of the 27-kDa intermediate was most susceptible to inhibition by a thiol protease inhibitor, p-chloromercuribenzoate. A thiol protease with a specificity for paired basic amino acids has been implicated in the processing of proenkephalin (18) although this enzyme, unlike the activity responsible for cleavage of the 27-kDa intermediate of proenkephalin, was inhibited by leupeptin, an inhibitor of lysosomal cathepsin B like enzymes. Some inhibition of cleavage of the 27-kDa intermediate was also observed with inhibitors of serine proteases. A trypsin-like enzyme which is capable of generating enkephalins from EC proteins (7, 8) has been purified from chromaffin granule lysate by affinity chromatography using immobilized soybean trypsin inhibitor. It is not clear from the

**DISCUSSION**

In previous work we showed that an antiserum to a synthetic peptide corresponding to residues 95–117 of bovine proenkephalin recognizes all the major intermediates of proenkephalin in bovine adrenal medulla (14). The specificity of this antiserum has enabled us to study the stability of the major intermediates of proenkephalin, corresponding to 27-, 20.5-, and 16.5-kDa EC proteins during incubation of intact and hypotonic lysates of bovine adrenal medullary chromaffin granules. This approach has the advantage that it enables the effect of endogenous enzymes on intermediates present in the tissue to be studied.
Although proenkephalin contains a possible site for Asn-proenkephalin, multiple species of synenkephalin, a fragment of two forms with the same COOH-terminal sequence (24). It has been suggested that a minor proportion of intermediates of similar molecular weight but with different amino acid sequences in prohormones has been isolated from bovine pituitary secretory granules (19).

Preliminary evidence was found for a regulatory mechanism affecting the processing of the 27-kDa proenkephalin intermediate that appeared to be dependent on the concentration of soluble factors in chromaffin granules (Fig. 4). This may be due to either (a) the presence of endogenous protease inhibitors or (b) the interaction of proenkephalin intermediates with components that provide some protection from proteolysis. Such a regulatory mechanism may be opposed by mechanisms for increasing the proteolytic processing following appropriate stimulation. Recent work has shown that a carboxypeptidase B-like enzyme can be selectively regulated by agents that increase enkephalin synthesis in cultured bovine adrenal chromaffin cells (20). It has also been shown that electrical stimulation results in both increased levels of proenkephalin mRNA and increased levels of enkephalin peptides (21).

We have recently shown that the 27-kDa intermediate of proenkephalin is associated with bovine chromaffin granule membranes (22). It is interesting to note that this is the major species associated with membranes, whereas the 20.5- and 16.5-kDa components are dominant in the soluble lysate fraction. The stability observed for both membrane-associated and soluble intermediates in intact granules is consistent with the slow processing of proenkephalin. Membrane-associated proenkephalin also appeared stable during incubation of chromaffin granule membranes, suggesting that the interaction of proenkephalin with membrane components provides some protection from cleavage. It is evident, however, that some soluble 27-kDa immunoreactivity is released into the buffer during incubation of chromaffin granule membranes. Unlike material that remained associated with the membranes, the soluble 27-kDa component appeared to be rapidly cleaved. It is not clear whether the processing enzyme responsible remained membrane-associated or was also present in a soluble form. The results are consistent with cleavage of the 27-kDa component and processing via 30.0- and 20.5-kDa intermediates to the 16.5-kDa component. The stability of the 20.5-kDa intermediate in these incubations was not as apparent as found in lysate experiments, possibly due to the absence of interactions with other components in the chromaffin granule lysate. Further studies would be required to confirm these results either using the purified 27-kDa intermediate or biologically synthesized studies employing pulse-chase experiments.

Analysis of hypotonic lysates by two-dimensional gel electrophoresis provided evidence for the presence of considerable microheterogeneity among intermediates in the processing of proenkephalin. Multiple species of synenkephalin, a fragment representing the NH2-terminal 72 residues of proenkephalin, have been reported (23), and we have isolated two forms of the 16.5-kDa component which have the same NH2-terminal sequence. It has been suggested that a minor proportion of a 18.2-kDa intermediate is glycosylated because of the presence of two forms with the same COOH-terminal sequence (24). Although proenkephalin contains a possible site for Asn-linked glycosylation, -Asn122-Ser198-Ser272, no glucosamine, galactosamine, mannose, fucose, galactose, xylose or glucose could be detected in hydrolysates of proenkephalin fragments containing this sequence (25). It is possible that the presence of intermediates of similar molecular weight but with different isoelectric points may be due to differences in processing resulting in some intermediates with residual basic amino acids at the COOH terminus. Other post-translational modifications, for example phosphorylation, may also account for the multiple charge forms seen in the present study. It is unlikely that the multiple species seen are due to artifacts as the results were consistent between experiments, and significant quantities of the individual species are present whereas only minor amounts would be expected if they resulted from sample preparation. Furthermore, the number of forms of other chromaffin granule matrix proteins, e.g. the chromogranins as shown by Coomassie Blue staining (data not shown), were consistent with other reports (26).

The results of the present work enabled characterization of the intermediates of proenkephalin and an assessment of their stability to endogenous processing enzymes present in intact, soluble, and membrane components of bovine adrenal medullary chromaffin granules. It will be important to compare the intermediates of proenkephalin generated by purified proteases with the intermediates identified after reaction with anti-proenkephalin (35-117) serum. Further experiments would benefit by use of the 27-kDa intermediate as a substrate for processing enzymes, and future work should also consider the regulation of the processing of this prohormone. The microheterogeneity present in intermediates of proenkephalin indicates that further studies concerning the post-translational modification of proenkephalin are warranted.

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